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# *In vivo* and *in vitro* persistence of pyridyloxobutyl DNA adducts from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

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**The persistence of pyridyloxobutyl DNA adducts in lung and liver of F-344 rats treated with the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was investigated. The levels of these adducts were determined at various time points up to 4 weeks post s.c. injection of [5-$^3$H]NNK (0.8 mg/kg body wt). Maximal levels of the adducts were observed between 4 and 24 h in both tissues. The disappearance of the adducts from lung and liver DNA was multiphasic with initial half-lives of 50 and 38 h respectively. In both cases, detectable levels of the pyridyloxobutyl adducts were observed at 4 weeks post injection. The *in vitro* rate of adduct disappearance was studied using calf thymus DNA reacted with 4-(acetoxymethylnitrosamino)-1-(3-[5-$^3$H]pyridyl)-1-butanone in the presence of esterase. Adduct levels were measured for up to 2 weeks after the initiation of the experiment. The decomposition of these adducts was triphasic with half-lives of 6, 120 and 430 h. The multiphasic disappearance of the pyridyloxobutyl adducts suggests that there is more than a single adduct generated upon pyridyloxobutylation of DNA and that at least one of these adducts has a significant lifetime in DNA.**

## Introduction

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) is an important carcinogenic constituent of tobacco and tobacco smoke. NNK induces a high incidence of lung, liver and nasal cavity tumors in F-344 rats (1). The carcinogenic activity of NNK is believed to be associated with its ability to be activated to DNA-reactive species (2,3) (Figure 1). Methylene hydroxylation leads to the formation of methanediazohydroxide, which causes DNA methylation *in vivo* (2). Methyl hydroxylation generates 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide, which pyridyloxobutylates DNA and hemoglobin (3,4). The pyridyloxobutyl adducts are unstable and can be released from DNA and hemoglobin upon hydrolysis as 4-hydroxy-1-(3-pyridyl)-1-butanone (4-HPB) (3,4). Treatment of rodents with another tobacco-specific nitrosamine, *N'*-nitrosonornicotine (NNN), also leads to the formation of these adducts (3,4).

We have developed a dosimetry assay for the measurement of 4-HPB released from the hemoglobin and DNA of tobacco users (5,6). The HPB-releasing adducts were detected in hemoglobin and DNA isolated from smokers, demonstrating that these individuals were capable of metabolically activating tobacco-specific nitrosamines. There were marked interindividual variations in adduct levels (5,6). In addition to the individual's capability to activate the nitrosamines, the adduct level will be affected by the amount and frequency of nitrosamine exposure as well as the lifetime of the adduct. The 4-HPB-releasing adducts of hemoglobin have been shown to persist in F-344 rats with a half-life of 9.1 days (4). Little is known about the lifetime of pyridyloxobutyl adducts in DNA. This knowledge would assist analysis of the levels observed in DNA of tissues from smokers and non-smokers.

Therefore, we undertook studies to determine the persistence of the 4-HPB-releasing DNA adducts in F-344 rat liver and lung following a single low dose of NNK. The *in vivo* stability of the adducts are compared to their lifetime *in vitro*.

## Materials and methods

*Chemicals and biochemicals*

[5-$^3$H]NNK (sp. act. 1.5 Ci/mmol) was obtained from ChemSyn Science Laboratories (Lenexa, KS). It was diluted, if necessary, with unlabeled synthetic NNK (7) to the required specific activity. 4-(Acetoxymethylnitrosamino)-1-(3-[5-$^3$H]pyridyl)-1-butanone ([5-$^3$H]NNKOAc), 4-HPB, and 3-hydroxy-1-(3-pyridyl)-1-butanone (3-HPB) were prepared as previously reported (7–9). Calf thymus DNA and porcine liver esterase were purchased from Sigma.

*Animal treatments*

Male F-344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY) and were housed under standard conditions as previously described (10). Eight groups of three rats (average weight 250 g) were given single s.c. injections of [5-$^3$H]NNK in saline. The animals were killed at the following time points: 4, 12, 24, 48, 96 h, 1, 2 and 4 weeks.

The rats in the first four groups received 0.81 mg/kg [5-$^3$H]NNK (1.0

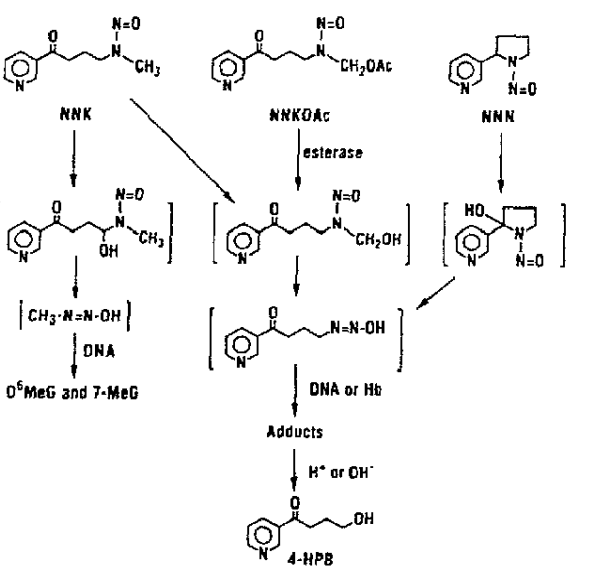


**Fig. 1.** Proposed activation routes of NNK, NNKOAc and NNN to pyridyloxobutylating species.

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; 3-HPB, 3-hydroxy-1-(3-pyridyl)-1-butanone.

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Ci/mmol). The remaining groups were treated with 0.85 mg/kg (1.1 or 1.5 Ci/mmol). At the appropriate time, blood was withdrawn from each animal by cardiac puncture under halothane anesthesia. The rats were then killed by decapitation and the lungs and livers were removed and frozen at −80°C. DNA was isolated from the tissues using a modification of the Marmur method (3).

*Analysis of DNA for levels of pyridyloxobutylation*

This was described previously (3,8,11,12). These studies have shown that strong acid hydrolysis (0.8 N HCl, 6 h, 80°C) of highly purified DNA releases 4-HPB. In the present study, 3−4 mg of DNA was used for each determination. The acid hydrolysates were neutralized, spiked with standards and separated on a C18 reverse-phase column (Waters C18 μBondapak 10 μ, 4.6 mm × 25 cm) with solvents A (20 mM sodium phosphate buffer, pH 7) and B (95% methanol, 5% $H_2O$) using a linear gradient from 100% A to 65% A over 60 min (flow: 1 ml/min). The levels of 4-HPB were determined from the radioactivity that co-eluted with the standard, using a Flo-one/Beta radioflow detector (Radiomatics Instruments, Tampa, FL). The levels of guanine present in the hydrolysates were determined by HPLC analysis (2) and the level of adduct was expressed as pmol released/μmol guanine. The percentage adduct remaining in DNA was determined by dividing the levels of adduct observed by the level present at 24 h and multiplying by 100. The 24 h time point was chosen as 100% since no new adduct formation seemed to occur after this point in both tissues.

To confirm that the radioactivity in the 2 and 4 week liver DNA hydrolysates was [5-$^3$H]4-HPB, the corresponding fractions were collected and concentrated to 1 ml under nitrogen. Sodium borohydride (3−5 μmol) was added to each sample. After 2 h at 20°C, the reaction was terminated by addition of 1 N HCl. The samples were then analyzed by reverse-phase HPLC (Phenomenex C18 Bondclone column) using solvents C (60 mM acetic acid) and D (methanol) at a flow rate of 1 ml/min. The reaction mixture was eluted with either an isocratic system (92% C and 8% D) or with a gradient from 100% C to 40% C/60% D over 60 min. In each case, the radioactivity now co-eluted with the reduced 4-HPB standard, 4-hydroxy-1-(3-pyridyl)-1-butanone

*In vitro DNA binding experiments*

[5-$^3$H]NNKOAc (0.5 mM, 66.4 μCi/ml) and calf thymus DNA (2 mg/ml) in 15 mM sodium citrate buffer, pH 7.0, were incubated for 1 h with porcine liver esterase (0.126 mg/ml) at room temperature. After 1 h, 2 M NaCl (1 ml) was added and the DNA was precipitated with ethanol, washed extensively with ethanol and dried under a nitrogen stream.

The DNA (23 mg) was redissolved in 100 mM sodium phosphate buffer, pH 7.4 (23 ml). The DNA solution was divided into two portions and each solution was incubated at 37°C. Aliquots (0.65 ml) were removed at 0, 0.5, 1, 1.5, 2, 4, 27, 51, 72 h, 1 and 2 weeks. The DNA was precipitated with ethanol after the addition of 2 M NaCl (0.5 ml). The DNA was washed extensively with ethanol, dried under a nitrogen stream and stored at −20°C. The levels of [5-$^3$H]4-HPB were measured as described above. However, these samples were analyzed using a linear gradient from 100% to 70% A over 30 min followed by a 10 min gradient to 50% A. 4-HPB eluted at 42 min under these conditions. The percentage of 4-HPB-releasing adducts remaining in DNA was determined by dividing the adduct level at the various time points by the amount initially present in the DNA and multiplying by 100.

The identity of [5-$^3$H]4-HPB was confirmed by co-chromatography with 4-HPB standard using a normal phase system. Two of the acid hydrolysates were neutralized with 1 N NaOH, spiked with unlabeled 4- and 3-HPB, and extracted with methylene chloride. The extracts were analyzed on an EM LiChrosorb Si-60, 10 μm column, 250 mm × 4.6 mm, using solvents A (100% ethyl acetate) and B (90% ethyl acetate and 10% methanol). A linear gradient from 100% A to 50% A over 20 min was used at a flow of 1 ml/min.

## Results

*In vivo studies*

Rats were treated with a single dose of [5-$^3$H]NNK and killed at various points following injection to determine the persistence of the HPB-releasing adducts *in vivo*. A dose of 0.8 mg/kg was chosen since it is a relatively low dose that is still expected to induce a significant number of lung tumors in rats after multiple injections (13). The DNA isolated from the lungs and livers of these rats was hydrolyzed in 0.8 N HCl and analyzed by reverse-phase HPLC to measure the levels of [5-$^3$H]4-HPB released. Representative radiograms of these hydrolysates are shown in Figure 2. [5-$^3$H]4-HBP was present in measurable quantities in all samples. The apex of the [5-$^3$H]4-HPB peak was between 3 and 20 times background in the 2 and 4 week DNA hydrolysates. The limit of detection was twice background. The identity of this

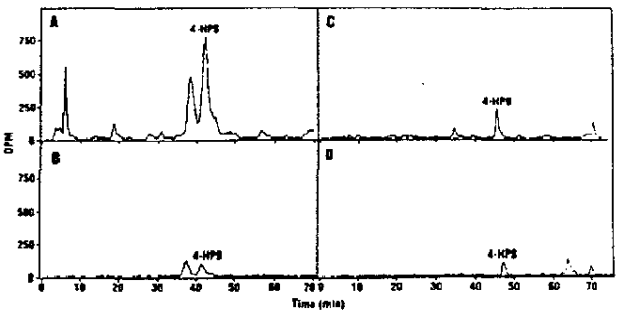


**Fig. 2.** Representative radiograms obtained upon reverse-phase HPLC analysis of acid hydrolysates of DNA isolated from liver (A and B) or lung (C and D) of rats treated with [5-$^3$H]NNK. The traces are from the 24 h (A and C) and 2 weeks (B and D) time points. In each case, 4-HPB was identified by co-elution with a standard. The radioactivity associated with the 4-HPB peak in the hydrolysates of the 2 week liver and lung DNA samples correspond to 972 and 996 d.p.m. above background respectively

**Table I.** Levels of [5-$^3$H]4-HPB released from lung and liver DNA isolated from rats treated with [5-$^3$H]NNK[a]

| Time | [5-$^3$H]4-HPB released (pmol/μmol guanine) | |
|---|---|---|
| | Lung[b] | Liver[c] |
| 4 h | 2.0 | 2.1 ± 0.8 |
| 12 h | 1.3 | 2.1 ± 0.3 |
| 24 h | 1.6 | 2.1 ± 0.1 |
| 48 h | 1.1 | 1.3 ± 0.7 |
| 96 h | 0.8 | 0.9 ± 0.2 |
| 1 week | 0.5[d] | 0.4[b] |
| 2 weeks | 0.2 ± 0.03[b,e] | 0.1 ± 0.1[e] |
| 4 weeks | 0.3[e] | 0.1 ± 0.01[e] |

[a]Groups of three rats received a single s.c. dose of [5-$^3$H]NNK and were killed at the appropriate time post-injection. The DNA was isolated from lung and liver using a modification of the Marmur method (2,3). Acid hydrolysates of the DNA (3−4 mg) were analyzed by reverse-phase HPLC linked to radioflow detection. See Materials and methods for details.
[b]Mean of two samples.
[c]Mean ± SD ($n$ = 3).
[d] Single sample
[e]The radioactivity co-eluting with standard 4-HPB in these samples ranged from 390 to 1800 d.p.m. above background.

radioactive peak as 4-HPB was determined by co-elution with synthetic 4-HPB. In addition, the 4-HPB peak in the 2 and 4 week DNA samples was collected, reduced with sodium borohydride, and found to co-elute with 4-hydroxy-1-(3-pyridyl)-1-butanol. This confirmed that the radioactive peak was indeed [5-$^3$H]4-HPB. There was a second major radioactive peak that was present in the liver DNA hydrolysates, eluting just prior to [5-$^3$H]4-HPB. The identity of this peak is not known and the amounts of this compound were variable.

The levels of 4-HPB measured in the rat lung and liver DNA hydrolysates are listed in Table I. The maximal levels of 4-HPB were observed between 4 and 24 h in both the lung and liver. In both tissues, measurable levels of the 4-HPB-releasing adducts were detected at 4 weeks following injection. The levels of 4-HPB did not differ greatly between the liver and lung. The rate of disappearance of these adducts was multiphasic in both tissues (Figure 3). The initial half-lives were 50 and 38 h in the lung and liver respectively.



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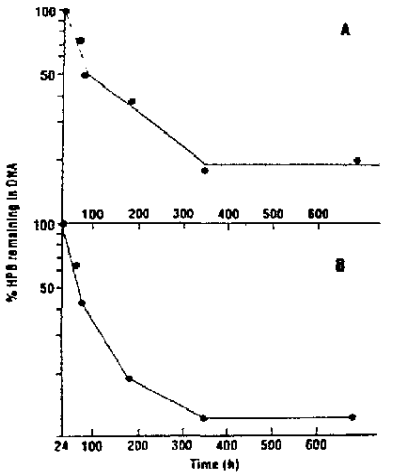


Fig. 3. *In vivo* stability of the 4-HPB releasing adducts in lung (A) and liver (B) DNA from rats treated with [5-$^3$H]NNK. The 24 h time point was chosen as 100%.

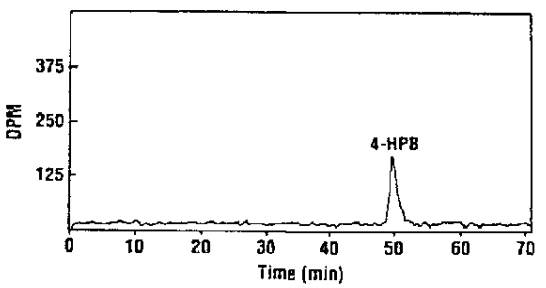


Fig. 4. A radiogram obtained upon reverse-phase HPLC analysis of an acid hydrolysate of calf thymus DNA following reaction with [5-$^3$H]NNKOAc. 4-HPB was identified by co-elution with a standard.

*In vitro DNA adduct stability studies*

NNKOAc generates the reactive pyridyloxobutylating agent *in situ* upon esterase hydrolysis (Figure 1). Calf thymus DNA was reacted with [5-$^3$H]NNKOAc in the presence of esterase to generate [5-$^3$H]pyridyloxobutylated DNA. All of the NNKOAc has been hydrolyzed during the 1 h reaction period. Strong acid hydrolysates of the alkylated DNA were analyzed by reverse-phase HPLC with radioflow detection. The radiograms of the hydrolysates displayed primarily a single radioactive peak that co-eluted with the 4-HPB standard (Figure 4). Analysis of methylene chloride extracts of the hydrolysates by normal-phase HPLC chromatography with radioflow detection confirmed that we were measuring 4-HPB and not 3-HPB. These two compounds co-elute in the reverse-phase HPLC system. Under the alkylation conditions, >94% of the HPB released from the DNA was 4-HPB. The level of 3-HPB increased with longer reaction times (data not shown). 3-HPB is released from DNA alkylated by the $\alpha,\beta$-unsaturated ketone, 1-(3-pyridyl)but-2-ene-1-one, a significant by-product in the solvolysis of NNKOAc (14). Since the $\alpha,\beta$-unsaturated ketone reacts more slowly than the diazohydroxide with DNA, we avoided the interference from this side reaction by shortening the DNA alkylation time.

The chemical stability of the 4-HPB releasing adducts was determined by incubating the alkylated DNA in pH 7.4 sodium

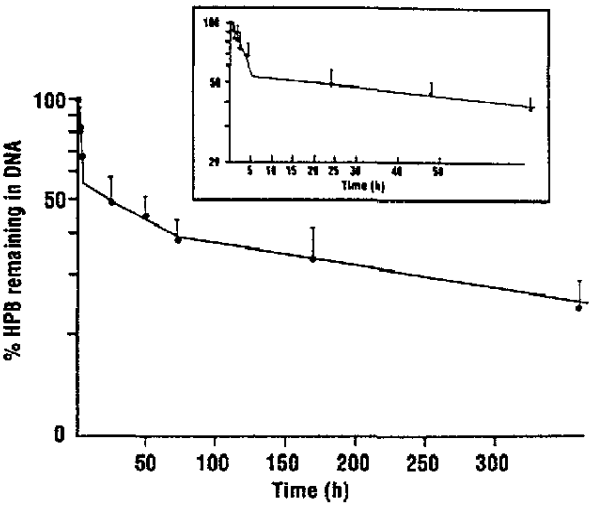


Fig. 5. *In vitro* stability of the 4-HPB-releasing adducts in calf thymus DNA at pH 7.4. The results presented are the average of five experiments. The error bars represent the standard error for each point.

phosphate buffer at 37°C and isolating and analyzing samples at intervals up to 2 weeks. The results are shown in Figure 5. The disappearance of the 4-HPB-releasing adducts appears to be triphasic with half-lives of 6, 120 and 430 h.

## Discussion

The persistence of 4-HPB-releasing DNA adducts in target tissues was determined following a single s.c. dose of [5-$^3$H]NNK (0.8 mg/kg) to F-344 rats. Injection of 0.3 mg/kg, s.c., three times weekly for 20 weeks, results in a 13% incidence of lung tumors in rats, whereas a dose of 1.0 mg/kg, following the same protocol, increases the tumor incidence to 48–53% (2,13). Therefore, the dose used in this study should induce a significant incidence of lung tumors after multiple injections. At this dose we found the HPB-releasing adducts to persist for up to 4 weeks in both lung and liver. It is not clear how chronic dosing would affect the persistence of these 4-HPB-releasing DNA adducts.

Many of the reports concerning NNK have focused on the formation, persistence and potential biochemical effects of the DNA methylation pathway in NNK-induced carcinogenesis. It has been well documented that the promutagenic $O^6$-methylguanyl residue is generated in tissues exposed to NNK and that this adduct persists in Clara cells (15). Based on reported data, the level of $O^6$-methylguanine in the lung expected for the dose used in this study would be ~0.5 pmol/μmol at 4 h (11,16). A recent study demonstrated that there was a strong correlation between the level of $O^6$-methylguanine accumulated in Clara cells of NNK-treated rat lungs and tumor formation (13). However, the NNK-induced lung tumors were not derived from this cell type, suggesting that other events, in addition to DNA methylation, are important in NNK carcinogenesis.

Relatively little is known about the *in vivo* biological effects of pyridyloxobutyl adducts. However, model compounds such as NNKOAc and 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone are potent mutagens in the Ames assay (L.A.Peterson and P.G.Foiles, unpublished results, 17). Furthermore, NNN,



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which is capable of pyridyloxobutylating but not methylating DNA (3), is a strong nasal cavity and esophogeal carcinogen (18,19). These observations suggest a potential role for pyridyloxobutyl adducts in the carcinogenic effects of NNK and NNN. The observation that these adducts have a significant lifetime in DNA suggests that they may be involved in tumor induction by these tobacco-specific nitrosamines.

The chemical structure of the 4-HPB-releasing adduct(s) is unknown. Their isolation and characterization have been confounded by their instability under the normal chemical or enzymatic DNA hydrolysis conditions (3,12). Our studies demonstrate that they are sufficiently stable in DNA to persist for up to 4 weeks. They could elicit potentially harmful biochemical effects during this time.

The disappearance of the 4-HPB releasing adducts from lung and liver DNA *in vivo* appears to be multi-phasic with the initial phases having half-lives of 50 and 38 h respectively. The *in vitro* disappearance of the adducts was triphasic with half-lives of 6, 120 and 430 h. The very short initial half-life was not observed in the *in vivo* DNA. Adduct formation in the *in vitro* studies happens within 1 h, whereas adduct formation *in vivo* is a more dynamic process that occurs over a longer time period. Adduct formation and decomposition or repair are occurring simultaneously *in vivo* and an unstable adduct could be lost. In addition, the isolation procedure of the tissue DNA is much longer than the *in vitro* DNA.

The multiphasic nature of the half-lives of the HPB releasing adducts in DNA suggests that there is more than one DNA adduct formed from 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide. Solvolysis studies of NNKOAc demonstrated that this diazohydroxide is capable of reacting with nucleophiles directly or via a cyclic oxonium ion to produce straight chain or cyclic adducts respectively (14). One might anticipate that these two adduct types would have different stabilities but both could decompose to 4-HPB under the hydrolysis conditions. Furthermore, there are multiple nucleophilic sites in DNA and reaction at each site would provide an adduct of differing lability. Alternatively, local DNA conformation or base sequence may also have an influence on adduct half-life.

In summary, we have observed that the pyridyloxobutyl adducts that are formed from NNK have significant half-lives *in vitro* and *in vivo*. This stability may have importance in the mechanism of NNK-induced carcinogenesis. These observations also suggest that these adducts should be detectable in the DNA of humans exposed to tobacco-specific nitrosamines for up to 4 weeks following their last exposure.

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